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Original Article

Bilosome Nanocarriers for Enhanced Oral Bioavailability and Cardiovascular Activity of Milrinone

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ABSTRACT

Background: Cardiovascular Diseases (CVDs) are the main causes of death in both industrialized and developing nations, which are mostly brought on by poor lifestyles and insufficient physical activity. Recent years many novel approaches have been developed for effective drug delivery of cardiovascular drugs. Milrinone [MRN] is a cardiovascular medication that is mainly used in the intensive care unit and cardiac unit to provide cardiac support to patients suffering from acute heart failure. It is also used to wear patients who have pre-existing left ventricular dysfunction from cardiopulmonary bypass, or it can be used as a temporising agent for patients awaiting heart surgery or transplantation. Milrinone is poorly water soluble and milrinone salt was used in the market. **Aim:** This work aimed to develop; a bilosome carrier was used to enhance the bioavailability of MRN. Materials and Methods By using thin film hydration method the MRN Bilosomes were prepared. **Results:** The formulation shows spherical morphology by SEM image and particle size were found to be 120 nm±6.8. The formulation was stable which shows -37.2±2.3 mV of zeta potential. The drug release was 88±1.3% with the Entrapment efficiency of 81±1.7% w/w. Cellular uptake and cell viability studies were carried using H9c2 cells. Flowcytometry technique was used for cellular uptake study. The results indicate the fluorescence seen for the uptake of MRN-bilosomes by the cells. The MTT assay indicates there was 2-fold increases in cell viability of MRN-bilosomes when compared to milrinone lactate. **Conclusion:** These findings suggest that MRN bilosomes were effective against cardiovascular disease with increased bioavailability.

Keywords: Milrinone, Bilosomes, Cardiovascular Disease, H9c2 Cells, Cellular Uptake Study.

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INTRODUCTION

Cardiovascular Disease (CVDs) are fatal disease in both industrialized and developing nations which affects the person leading unhealthy lifestyles and insufficient physical activity. More than 50% of CVD cases globally are caused by Congestive Heart Failure (CHF), which is characterized by a buildup of plaque in the coronary artery that obstructs the flow of blood to the heart and leads in permanent heart failure. This condition also causes cardiac necrosis. Cardiovascular Disease (CVD) is the major cause of morbidity and mortality among women in the United States, accounting for 23.0% of all deaths in 2017. To reduce the considerable impact of CVD on the health of women, reliable Cardiovascular Risk (CVR) screening indicators and highly sensitive prediction models must be developed.[1]

The leading global cause of death is thought to be cardiovascular disease. More than 80% of all CVD-related deaths take place in developing and underdeveloped nations. This is anticipated to drastically rise, especially in third-world nations like Ethiopia. The Cardiovascular system's main job is to transport away the metabolic waste products produced by all body cells and to ensure that they receive an adequate number of materials for their optimal function. The heart serves as the body's main pump in this well-organized transport system for blood, which circulates within a closed system with varying pressure gradients.[2] Physical activity significantly lowers the risk of cardiovascular events such stroke, heart failure and coronary heart disease.[3-7]

Heart Failure (HF), a significant public health concern, according to estimates, there will be more than eight million HF patients in the US by 2030 and the accompanying medical expenses will rise from $31 billion in 2012 to $70 billion in 2030.[8] Numerous researches has looked into the relationship between obesity and HF and they have linked negative metabolic changes and cardiac remodeling to the development of HF.[9]

Milrinone [MRN] (Figure 1) is frequently used in the intensive care unit and cardiac unit, MRN is used to provide cardiac

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support for patients suffering from acute heart failure, to wear patients who have pre-existing left ventricular dysfunction from cardio pulmonary bypass, or as a temporizing agent for patients awaiting heart surgery or transplantation. Milrinone is a member of the PDE III inhibitor medication class. It is used to treat cerebral vasospasm in SAH patients by increasing intracellular CAMP concentration and providing a large calcium influx for positive inotropic effects.[10,11]

Bilosomes are a kind of vesicular carrier that are created when bile acid salts are fused into the bilayer membrane of non-ionic surfactant molecules. They have a higher penetration capacity since they are pliable, soft carriers. They improve their absorption and, as a result, bioavailability when loaded with medicinal substances. Moreover, they are nontoxic and have no known toxicity, making them appropriate for use in drug delivery.[12]

Bilosomes or liposomes are nanoparticles with bile salt integrated into them that may or may not contain cholesterol. Because bile salts can withstand the harsh GIT environment and protect the entrapped drug, bilosomes are physically and chemically stable when compared to more conventional vesicles (liposomes and noisome).

The current available MRN formulation should be taken orally three to four times a day. To overcome the major drawbacks of MRN and to enhance its efficiency bilosomes were taken into consideration. The major ingredient of bilosomes is bile salts, which regulates the cholesterol level in the body. Hence, in this work, we planned to prepare MRN bilosomes using bile salts to enhance its bioavailability through controlled release.

MATERIALS AND METHODS

Preparation of Bilosomes

Thin film hydration technique was used in the process of making Bilosomes. Cholesterol and Span 60 were dissolved in Chloroform and Methanol. The solution kept in rotary evaporator at 50°C to form a thin film. Milrinone was dissolved in DMSO and add bile acid into phosphate buffer 7.4 pH and mixed well. Stirring was maintained for 30 min using magnetic stirrer and kept in bath sonicator for 3 hr under 37°C. The blends were centrifuged under 2000 rpm for 20 min and the pellets were freeze dried using lyophilizer.

Characterization

Ultra Violet-Spectroscopy

From the stock solution serial dilution of 10, 20, 30, 40 and 50 µg/mL was prepared and absorbance values were measured at 356 nm with methanol as blank.[13]

Fourier Transform Infrared (FTIR)

MRN compatibility with bilosomal ingredient was analyzed. The FTIR spectra of pure MRN, SPAN 60, SPC and SDC were recorded using IR Spectroscopy.[14]

Zeta Sizer

Zeta sizer Nano ZS (Malvern Instruments) was used to determine the bilosomes by Dynamic Light Scattering (DLS). In short, to achieve an effective light scattering intensity, the samples were diluted 15 times with deionized water and the temperature was then kept at 25°C. Three averages of at least ten runs of measurements are included in the results.[15]

Zeta Potential

Zeta sizer Nano ZS (Malvern Instruments) was used to measure the zeta potential of the produced nanocarriers suspended in 0.25% (w/v) saline solution (pH 7.4, conductivity 2.0 ms/cm). The diluted sample was used to analyses it utilizing the electrophoretic mobility of the particles in an electrical field. The average of three runs is used to obtain the results.[15]

Encapsulation Efficiency

Below formula was used to calculate Encapsulation efficiency (EE %):

$$EE\ \% = (Tw/Aw) \times 100\%$$ [16]

Where Tw is the total amount of the incorporated material and Aw is the actual quantity of material added initially during the preparation. Tw and Aw can be determined using spectroscopic method.[13]

Scanning Electron Microscopy

Scanning Electron Microscopy (SEM) was used to determine the external surface morphology of bilosome nanoparticles. The samples were adhered to an aluminium stub using double-sided tape. The stub was securely fastened with tape and the lyophilized sample was gently distributed throughout its surface. To guarantee the conductivity of the sample, a thin layer of gold was sputter coated onto the sample stub using a JFC 1200 fine coater (Japan).

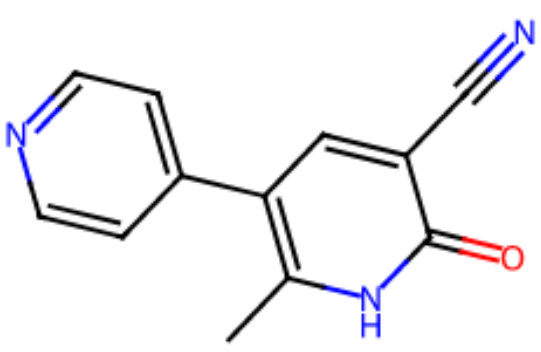

Figure 1: Milrinone.

SEM examination was performed on this treated sample in order to determine the exterior surface morphology.[17]

Drug Release

The drug release was carried out by using dialysis membrane. pH 7.4 Phosphate buffer saline was prepared and taken in a beaker. The formulated bilosomes were placed into a dialysis membrane and knotted. The dialysis membrane containing formulated bilosomes were dropped into beaker containing PBS and maintained at 37°C under stirring. The samples were withdrawn at regular time intervals and the drug content was measured using UV.

Cytotoxicity and Cellular Uptake Study

The cytotoxicity and cellular uptake analysis of MRN nanoparticles on H9c2 cells was performed and stated in 1983 by Mosmann with slight modification. Using a hemocytometer, the cells were counted, diluted in DMEM media to a density of 1104 cells/mL, then added to individual wells of 96-well plates and allowed to remain for 24 hr. Each well was treated with the control and various concentrations of nanoparticles ranging from 100 to 1.953 µg/mL after the L6 cells had been treated. H9c2 cells are cultured about 24 hr at 37°C in incubator. After flushed with pure culture fluid and MTT (5 µg/mL in PBS) dye the cells holding nanoparticles were then treated for a further 4 hr at 37°C.

The fluorescence intensity was calculated using spectrophotometer at a range from 489 nm to 535 nm. The data were presented as a percentage of stable cells depend on the control. The half-maximum Inhibitory Concentration (IC_{50}) values are calculated and the ideal doses were evaluated over time.[18,19]

RESULTS

UV Spectroscopy

The amount of UV or visible light that a sample either transmitted through or absorbed as compared to a blank or reference sample. The sample composition affects this attribute, which may reveal details about the sample's number and quality. Serial dilutions of 10, 20, 30, 40 and 50 µg/mL were made from the stock solution, which results in linear graph with regression coefficient of 0.992. Hence using the UV absorbance, further analysis of MRN were carried out.

FTIR

Milrinone, SPAN 60, Soya Phosphatidylcholine and mixture of Milrinone and Bilosomes were measured FTIR and the spectra for each compound were given in of identification of functional groups were given in Figures 2-4. The absorption curve was obtained by the technique on process of emission or absorption of the solids and liquid particles. The mixture of the compounds was measured using FTIR.

The spectral data obtained for the drug Milirnone are 2222.74 cm^{-1} (C-N), 3640.30 cm^{-1} (NH), 1667.18 cm^{-1} (C=O), also spectra 1386.02 cm^{-1} (CH_3) and 3725.92 cm^{-1} (N).

The peaks of bilosomes have functional groups of excipients and drug. The spectra 2921.63 cm^{-1} was obtained for the functional group OH and other peak obtained were 1747.1 cm^{1} (C=O), also spectra of 2029.77 cm^{-1} (O-H), alkaline group peak was at the range 1516.35 cm^{-1}. This indicates the there was no incompatibility found between drug and the excipients.

Particle Size analysis

The size of bilosomes was measured using Malvern zeta sizer. The size was measured thrice to found the average size of the prepared

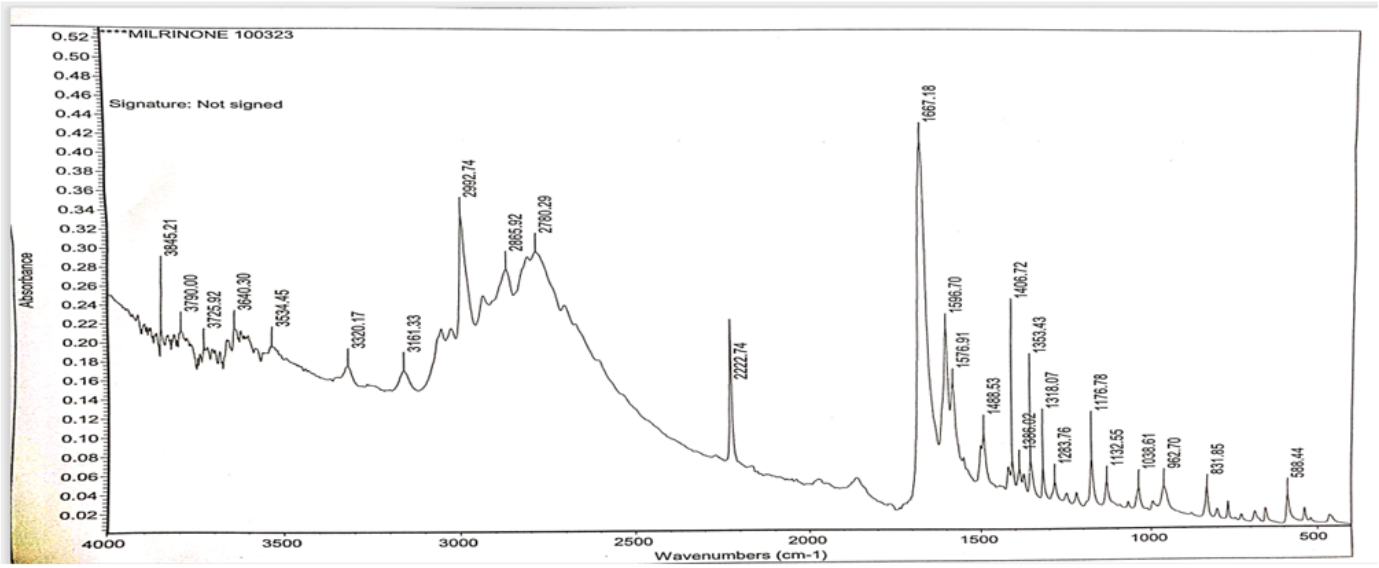

Figure 2: FTIR spectrum of Milirnone.

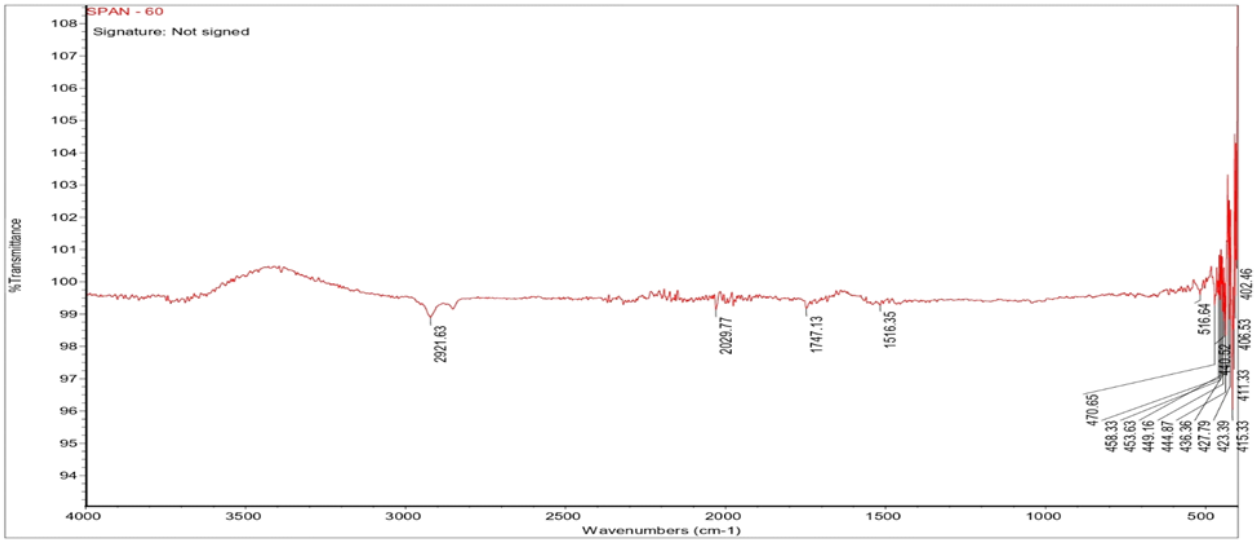

Figure 3: FTIR spectrum of excipients.

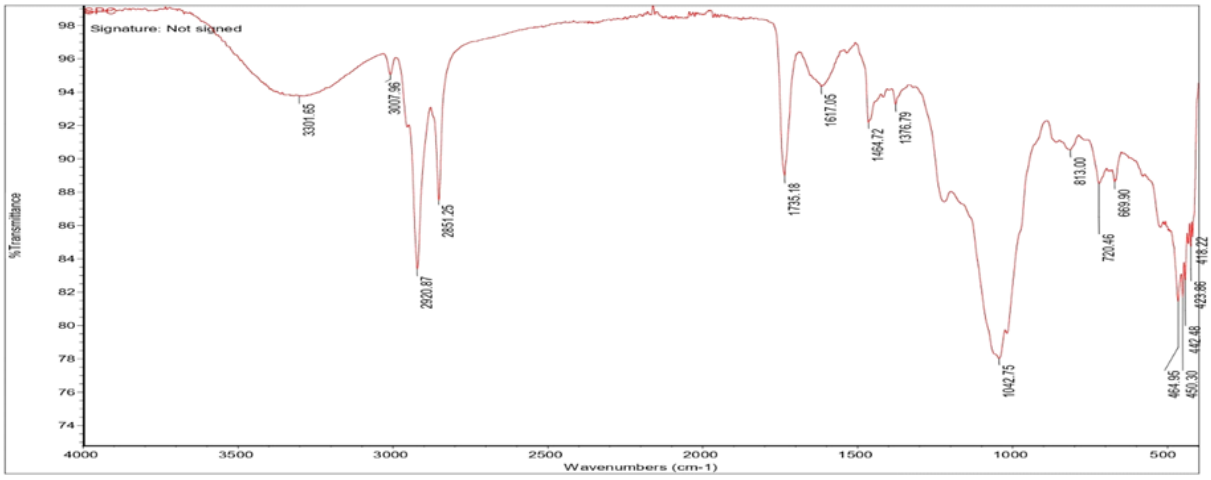

Figure 4: FTIR spectra of bilosome.

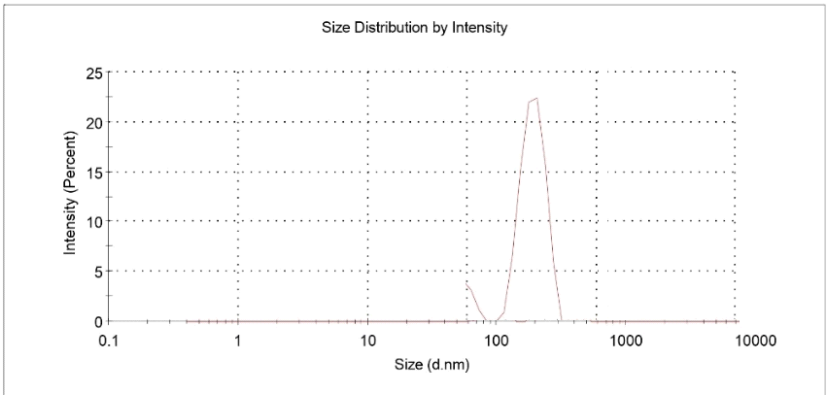

Figure 5: Size Distribution graph of MRN bilosome.

MRN bilosomes (Figure 5) and it was found to be 120±6.8 nm. This indicates the prepared formulation is in nano size which will overcome the solubility issue of MRN.

Zeta Potential

The zeta potential value was found to be -37.2 mV (Figure 6). This indicates the prepared MRN Bilosomes were stable and do not showed aggregates.

Encapsulation Efficiency (EE)

The Percentage EE within bilosomal formulae ranged from 80 to 88% hence justifies the encapsulation of MRN with bilosomes vesicles, thus MRN can be carried through bilosomes is evident.

Scanning Electron Microscope

The Scanning Electron Microscope images shows the preparation of bilosomes were spherical in shape. The images (Figure 7) indicate there is no accumulation of particles and the morphology was clearly shown under 10000X magnification.

Drug Release

The MRN bilosome formulation was tested for *in vitro* release during a 12 hr period at 37°C in buffer media with a pH of 7.4. Drug released was estimated with UV analysis. A graph of medication release % against time was computed and plotted. The plot indicates initial burst release from bile carrier. After 4 hr there was a steady release which indicates the controlled release from the formulation. Maximum release was found at 12th hr with 88±1.3% (Figure 8).

Cytotoxcity And Cellular Uptake Study

Cellular uptake and cell viability studies were carried using H9c2 cells. In order to assess both efficiency and safety of the prepared bilosome, cytotoxicity study was carried. The cells were treated with MRN, MRN BLS and hypoxia conditions with different

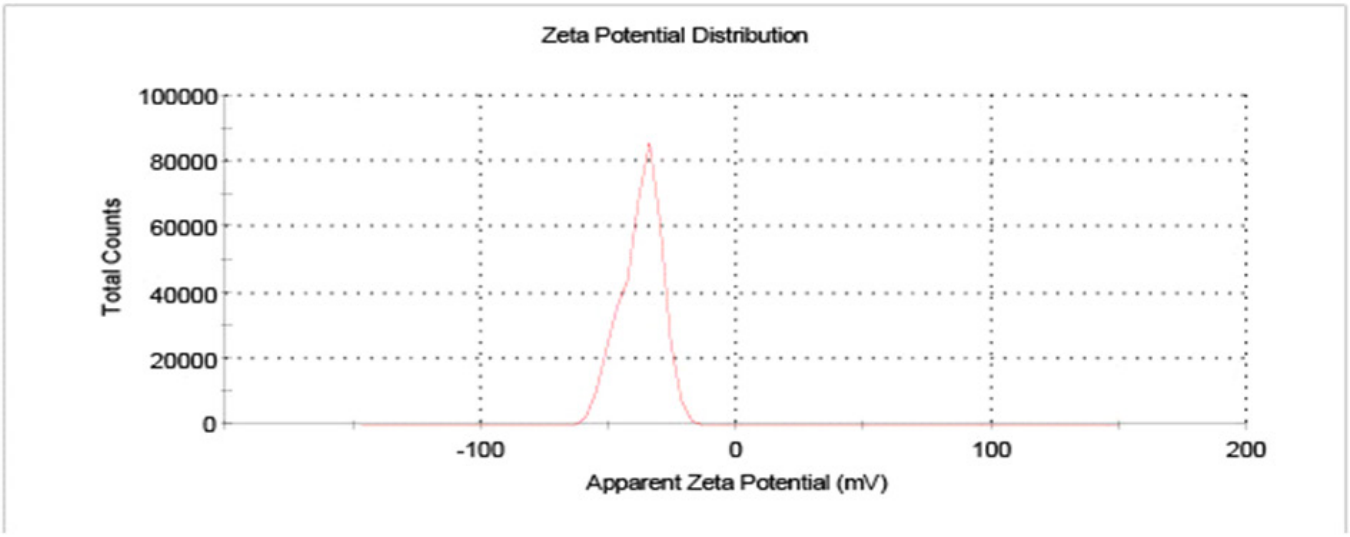

Figure 6: Zeta potential of MRN bilosomes.

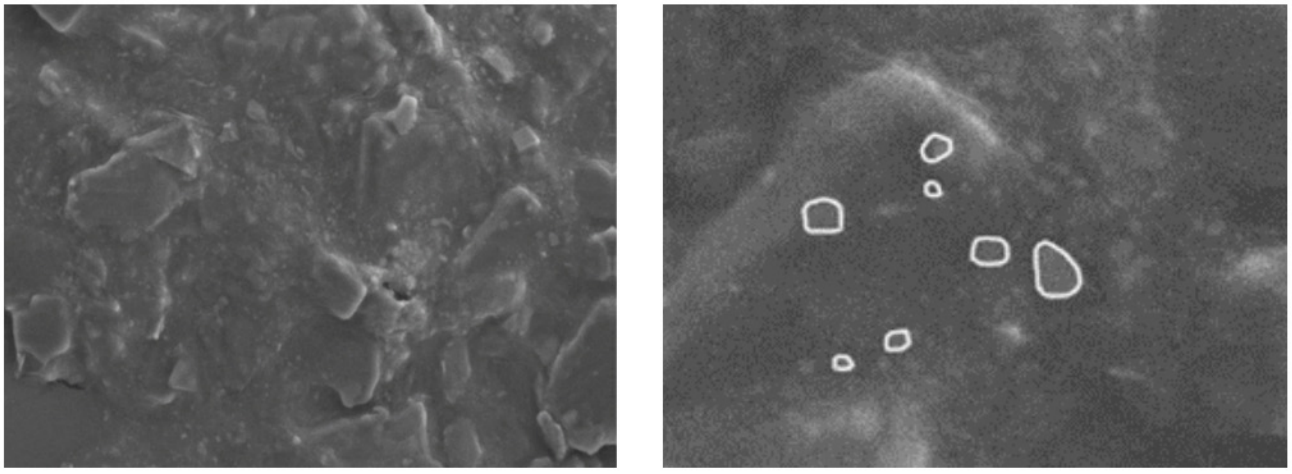

Figure 7: SEM image of MRN Bilosome.

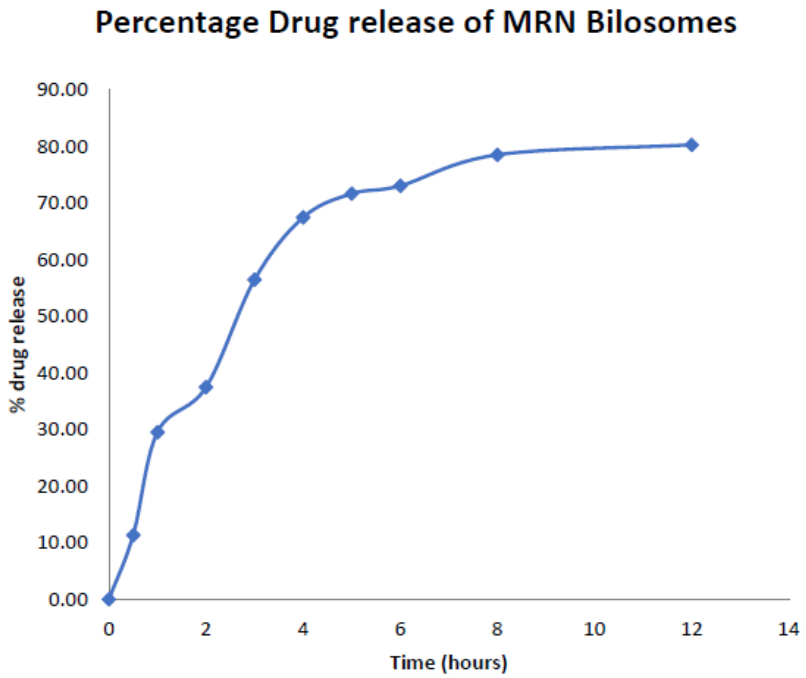

Figure 8: Percentage release of MRN from the MRN Bilosomes.

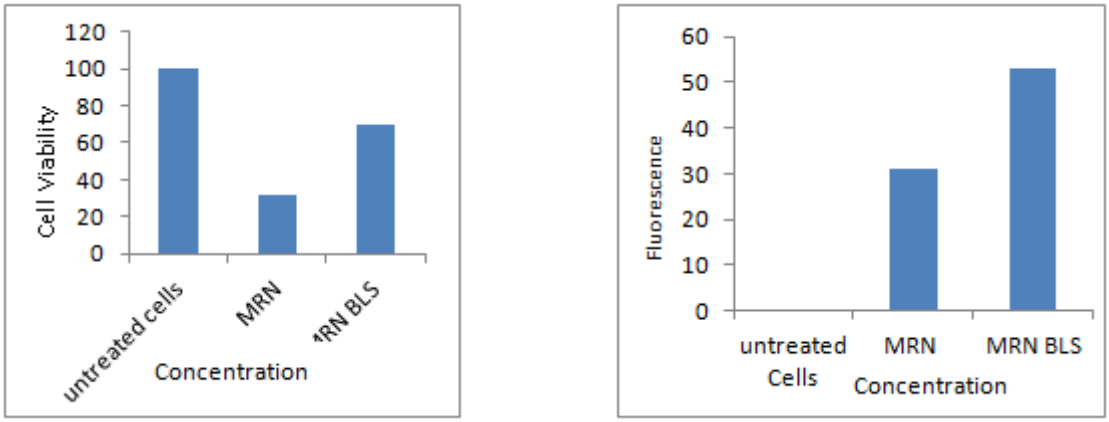

Figure 9: (a): Cytotoxicity study of MRN and MRN bilosomes; (b): Cell Uptake study of MRN and MRN bilosomes.

concentrations for 48 hr. The results stated that percentage cell viability of MRN bilosomes shows 69.7±1.9% and MRN shows 32.9±2.9%. The percentage cell viability indicates there was 2-fold increases in activity of bilosomes when compared to pure milrinone. Same way there was an increase level of calcium in the hypoxia cells and leads to cell death (Figure 9a).

The cellular uptake study was carried out to ensure the uptake of bilosomes by flow cytometry and the results indicate the fluorescence seen for the uptake of MRN-bilosomes by the cells. Deep red labels FITC-PI kit was used for estimation of cell uptake of drug and bilosomes which was compared with untreated cells. The pure drug MRN shows 31% of cells exhibited fluorescence whereas bilosomes were taken by 53% of cells. Hence the results indicate the bilosome formulation was validated for intercellular uptake of H9c2 cells (Figure 9b).

DISCUSSION

Bilosomes were prepared using thin film hydration technique to encapsulate Milrinone to overcome solubility issue. Initially UV, FTIR studies were carried out to check the characterization of drug along with the excipients. The IR spectrum of the compounds indicates the distinctive peaks of functional groups of drug and excipient in the formulation which facilitate the compatibility of the compounds. Hence, the formulation was carried out with further analysis to ensure the particle size, stability, morphology and encapsulation efficiency. The zeta size results of formulation indicates that the particle size was within the nano range (120 nm) and the stability of the formulation was determined by zeta potential, which shows -37.2 mV. The results ensure that particles were discrete and stable. Morphology was determined by SEM analysis and entrapment efficiency was found to be 80 to 90% w/w.

In vitro drug release from the formulation was performed using dialysis membrane for 12 hr at pH 7.4. The release percentage was calculated and found that initially there was burst and further the release was controlled. To ensure the cardiovascular activity of Milrinone *in vitro* cytotoxicity and cellular uptake was performed in H9c2 cells. There was a 2-fold increased activity in MRN bilosomes when compared to MRN.

CONCLUSION

Milrinone, a cardiovascular drug, which belongs to BCS class II. To overcome the drawbacks of milrinone, nano formulation approach was done. The bile salts were choosen as nano carrier which is known as bilosomes. The MRN Bilosomes were prepared using thin Film hydration method. From the results it clearly indicates that the prepared bilosomes were stable and increase the cell viability. The cellular uptake levels of bilosomes were also high when compared to native MRN. Hence, MRN can be formulated into bilosomes for enhancing its solubility and bioavailability.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

MRN: Milrinone; **MRN BLS:** Milrinone Bilosome; **SEM:** Scanning Electron Microscope; **FITC:** fluorescein isothiocyanate; **PI:** Propidium iodide; **CVD:** Cardiovascular diseases, **CVR:** Cardiovascular risk; **HF:** Heart failure; **SAH:** Subarachnoid hemorrhage; **SPC:** soy phosphatidylcholine; **SDC:** Sodium Deoxycholate; **FTIR:** Fourier-transform infrared; **UV:** Ultraviolet spectroscopy; **EE:** Encapsulation Efficiency; **IC_{50}:** Half maximal inhibitory concentration; **DMEM:** Dulbecco's Modified Eagle Medium; **DMSO:** dimethyl sulfoxide, **GIT:** Gastrointestinal tract; **CAMP:** Cyclic Adenosine MonoPhosphate.

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